Immunization with LJM11 salivary protein protects against infection with *Leishmania braziliensis* in the presence of *Lutzomyia longipalpis* saliva

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ABSTRACT

Leishmania is transmitted in the presence of sand fly saliva. Protective immunity generated by saliva has encouraged identification of a vector salivary-based vaccine. Previous studies have shown that immunization with LJM11, a salivary protein from *Lutzomyia longipalpis,* is able to induce a Th1 immune response and protect mice against bites of *Leishmania major*-infected *Lutzomyia longipalpis*. Here, we further investigate if immunization with LJM11 recombinant protein is able to confer cross-protection against infection with *Leishmania braziliensis* associated with salivary gland sonicate (SGS) from *Lutzomyia intermedia* or *Lu. longipalpis*. Mice immunized with LJM11 protein exhibited an increased production of anti-LJM11 IgG, IgG1 and IgG2a and a DTH response characterized by an inflammatory infiltrate with the presence of $CD4^+$ $IFN-\gamma^+$ T cells. LJM11-immunized mice were intradermally infected in the ear with *L. braziliensis* in the presence of *Lu. longipalpis* or *Lu. intermedia* SGS. A significant reduction of parasite numbers in the ear and lymph node in the group challenged with *L. braziliensis* plus *Lu. longipalpis* SGS was observed, but not when the challenge was performed with *L. braziliensis* plus *Lu. intermedia* SGS. A higher specific production of IFN-γ and absence of IL-10 by lymph node cells were only observed in LJM11 immunized mice after infection. After two weeks, a similar frequency of $CD4^+$ $IFN-\gamma^+$ T cells was detected in LJM11 and BSA groups challenged with *L. braziliensis* plus *Lu. longipalpis* SGS, suggesting that early events possibly triggered by immunization are essential for protection against *Leishmania* infection. Our findings support the specificity of saliva-mediated immune responses and reinforce the importance of identifying cross-protective salivary antigens.

1. Introduction

Leishmaniasis is a vector-borne disease transmitted by infected female sand flies. During bloodfeeding sand flies deposit a combination of saliva, parasites and other parasite-derived factors into the skin of the vertebrate host (Ribeiro, 1987; Kamhawi et al., 2000; Rogers, 2012; Atayde et al., 2015). Constant exposure to bites of uninfected sandflies or immunization with salivary proteins is known to induce cellular and humoral immune responses (Oliveira et al., 2012; Oliveira et al., 2015; De Moura et al., 2013). Immunization with single sand fly salivary molecules induce a Leishmania-protective Th1 immune response encouraging the identification of potential vaccine candidates (Kamhawi et al., 2000; Morris et al., 2001; Gomes et al., 2008; Gomes et al., 2012; Tavares et al., 2011; De Moura et al., 2013; Oliveira et al., 2012; Oliveira et al., 2015).

The use of a salivary protein as an anti-*Leishmania* vaccine component has been extensively discussed (Oliveira et al., 2015; De Moura et al., 2013). However, the variety of salivary molecules between different species of vectors should be considered when developing a salivary-based vaccine (Volf and Rohousová, 2001; Rohousova et al., 2005; Rohousová et al., 2012; Hosseini-Vasoukolaei et al., 2016; Abdeladhim et al., 2016). In fact, immunity and protection resulting from immunization with salivary proteins was shown to be species-specific (Thiakaki et al., 2005; Drahota et al., 2014; Lestinova et al., 2015).

Fig. 1. Humoral immune response and delayed-type hypersensitiviy (DTH) response generataded by LJM11 recombinant protein in mice. BALB/c mice were immunized with LJM11 or BSA in the right ear. Fifteen days after the last immunization, before infection, sera from the animals (n = 10 per group) were collected for detection of total IgG, IgG1 and IgG2a anti-LJM11. Dotted line represents cut-off values (A). Ear tissue was analyzed 48 h after the last immunization for measurement of ear thickness (n = 10 per group) (B) and staining with hematoxilin eosin was performed to observe the cellular infiltration by light microscopy. Arrows indicate the inflammatory infiltrate. (C). Dot plots of one representative experiment showing frequency of CD4$^+$IFN-γ^+ T cells in the ear of mice (n = 5 per group) following challenge (D). Experiments were repeated three times.*p < 0.005 performed between LJM11 and BSA immunized groups at 48 h, ***p < 0.001 performed between LJM11 and BSA immunized animals.

Recently, LJM, a salivary protein from saliva of the sand fly *Lu. longipalpis*, was identified as an immunogenic molecule to humans, dogs and mice exposed to bites of *Lu. longipalpis* (Collin et al., 2009; Teixeira et al., 2010). LJM11 protein belongs to the "yellow" protein family present in the salivary gland of the *Lutzomyia* and *Phlebotomus* sand flies. The function of LJM11 has been established as a high affinity binder of pro-inflammatory biogenic amines (Xu et al., 2011). The ability of this molecule to induce a cellular immune response in vertebrates and further protection against *Leishmania* was demonstrated in recent studies. Development of a delayed-type hypersensitivity response and the presence of IFN-γ^+CD4$^+$T cells creating a Th1 environment is the mechanism underlying the LJM11-induced protective immune response (Gomes et al., 2008; Gomes et al., 2012). Importantly, immunization with LJM11 protein resulted in protection against *Leishmania major* infection transmitted by *Lutzomyia longipalpis*, highlighting the protective ability of this salivary molecule (Gomes et al., 2012).

In Brazil, American cutaneous leishmaniasis (ACL) is caused by *L. braziliensis* transmitted by *Lu. intermedia*, its main vector. ACL is characterized as a chronic localized lesion that can potentially progress to muco-cutaneous leishmaniasis (Marsden, 1986; Rosales-Chilama et al., 2015). Here, we investigate if immunization with LJM11 is able to induce a cross-protective response against *L. braziliensis* infection in the presence of either *Lu. longipalpis* or *Lu. intermedia* SGS (salivary gland sonicate). We used the BALB/c model of *L. braziliensis* infection that closely resembles many aspects of human infection (De Moura et al., 2005). Protective immunity was obtained only when parasites were inoculated with *Lu. longipalpis* but not with *Lu. intermedia* SGS suggesting that LJM11-induced protective immune response is vector specific.

2. Methods

2.1. Ethics statement

Animal work was conducted according to the Guidelines for Animal Experimentation of the Conselho Nacional de Controle de Experimentação Animal (CONCEA). The IGM-FIOCRUZ Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (CEUA-017/2013-IGM/FIOCRUZ).

2.2. Animals

BALB/c mice (females), 6–8 weeks of age were obtained from the animal facility at IGM/FIOCRUZ and were maintained under pathogen-free conditions.

2.3. Leishmania parasites

Leishmania braziliensis (MHOM/BR/01/788) promastigotes were cultured at 25 °C in Schneider's insect medium (Sigma Chemical Co.), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) (Sigma) at 23 °C for 5–7 days when the parasites reached the stationary-phase.

2.4. Sand flies and preparation of salivary gland sonicate (SGS)

Lu. intermedia sand flies were captured in Corte de Pedra (Bahia, Brazil) and morphologically identified according to the identification key proposed by Young and Duncan (Young and Duncan, 1994). *Lu. longipalpis* sand flies (Cavunge, Bahia), were reared at Laboratório de Imunoparasitologia (IGM, Fiocruz, Bahia). Salivary glands from *Lu. intermedia* (wild caught) and *Lu. longipalpis* females (5–7 day-old) were dissected and stored at −70 °C. Immediately before use, glands were
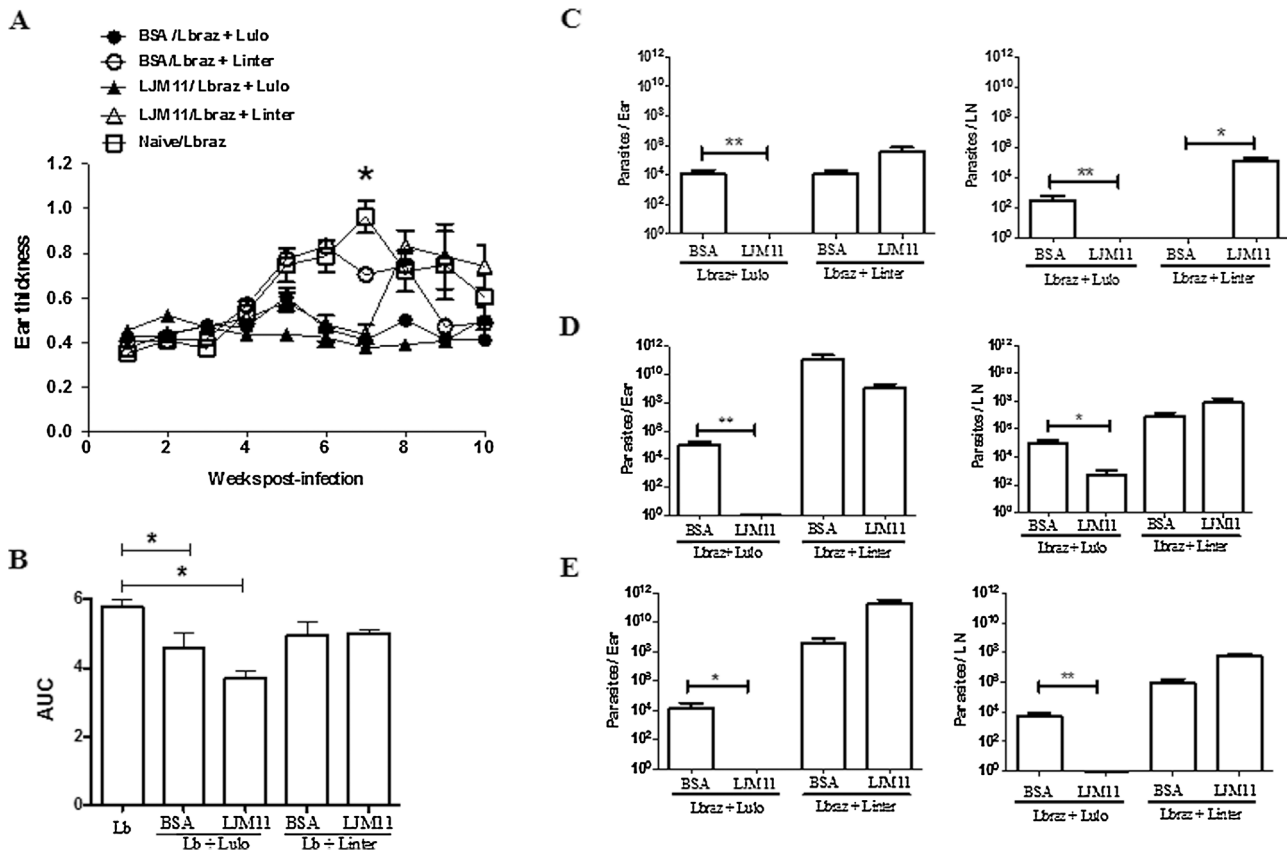
Fig. 2. Ear thickness and parasite load in LJM11 recombinant protein immunized mice following infection with *L. braziliensis* in the presence of *Lu. longipalpis* or *Lu. intermedia* SGS. BALB/c mice were immunized with LJM11 or BSA as a control. Two weeks after the last immunization mice were challenged with 10^5 *L. braziliensis* promastigotes in the presence of *Lu. longipalpis* (Lb + Lulo) or *Lu. intermedia* (Lb + Linter) SGS (n = 15 per group). The course of lesion development was monitored weekly (A), the area contained under the curves (AUC) obtained in A for each group was compared (B) *p < 0,0385 performed between Naïve and LJM11 or BSA/Lbraz + Lulo groups. Parasite load in the ear and draining lymph node, 2 (C), 5 (D) and 10 (E) weeks after infection (n = 5 per group). Each bar represents the mean and standard errors of the means., *p < 0,0385 ** p < 0.05. Experiments were repeated three times.

sonicated and centrifuged at 10,000*g* for 2 min to obtain the supernatant that was collected and transferred to a sterile tube and used for the studies. Protein concentration was determined by Bradford method (Bradford, 1976). The level of LPS contamination of SGS preparations was determined using the LAL Chromogenic Kit (QCL-1000, Lonza Bioscience). Results detected negligible levels of endotoxin in the salivary gland supernatants.

2.5. Immunization

BALB/c mice (n = 15 per group) were immunized intradermally three times in the left ear at 2-week intervals with 500 ng of either LJM11 recombinant protein or BSA (Sigma) in sterile saline solution (10 μl).

2.6. Expression and purification of LJM11 recombinant protein

LJM11 recombinant protein was produced and purified at the Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases. LPS levels were measured using the ToxinSensor Chromogenic LAL endotoxin assay kit (GenScript) and were below 20 EU/ml as previously described (Teixeira et al., 2010).

2.7. Determination of anti-LJM11 antibodies

Fifteen days after the last immunization, before infection, sera from immunized mice (n = 10 per group) were collected. Pre-immune sera was collected and used as control. ELISA plates (Maxisorp, Nunc) were

coated overnight at 4 °C with LJM11 (2 μg/ml) diluted in 0,1 M Na_2HCO_3 solution. After washing and blocking with 4% PBS-BSA for 2 h at room temperature, sera were diluted (1:50) and incubated for 1 h at 37 °C. After washing, plates were incubated for 1 h at 37 °C with alkaline phosphatase-conjugated anti-mouse IgG, IgG1 or IgG2a (1/1000) (Sigma). The reaction was developed with *p*-nitrophenylphosphate in sodium carbonate buffer pH 9.6 with 1 mg/ml of $MgCl2$. Absorbance was recorded at 405 nm.

2.8. Experimental infection

Two weeks after the last immunization with LJM11 or BSA, mice (n = 15 per group) were challenged intradermally in the left ear with 10^5 stationary phase *L. braziliensis* promastigotes in the presence of either (a) the equivalent of 1 pair of *Lu. intermedia* SGS (~1 μg of protein) (LJM11/Lbraz + Linter and BSA/Lbraz + Linter groups), or with (b) the equivalent of 0.5 pair of *Lu. longipalpis* SGS (~1μg of protein) (LJM11/Lbraz + Lulo and BSA/Lbraz + Lulo groups) (Tavares et al., 2011). Lesion size was measured weekly using a digital caliper (Thomas Scientific).

2.9. Limiting dilution assay (LDA)

Parasite load was determined 2, 5 and 10 weeks post-infection using the quantitative Limiting Dilution Assay previously described (Titus et al., 1985). Briefly, infected ears and draining lymph nodes (n = 5 per group) were aseptically removed from individual mice. Tissues were homogenized and diluted in Schneider's Insect Medium (Sigma)
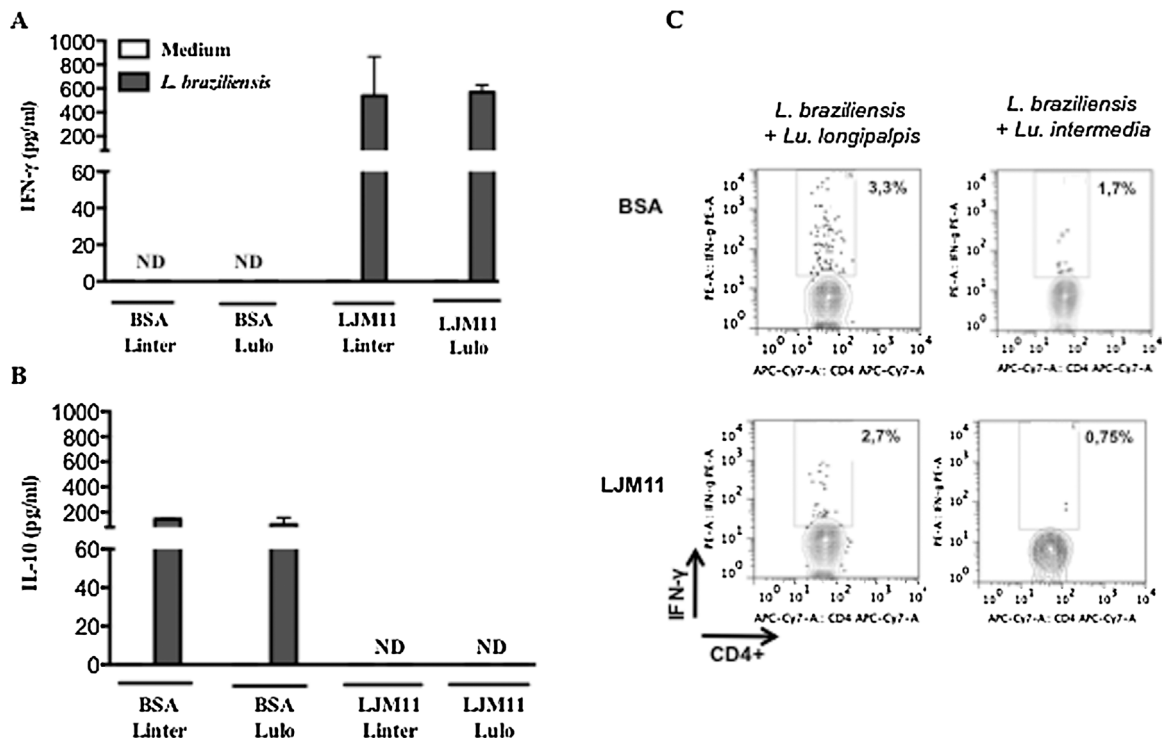
Fig. 3. Cytokine production by lymph node cells from mice immunized with LJM11 recombinant protein following infection with *L. braziliensis* in the presence of *Lu. longipalpis* or *Lu. intermedia* SGS. BALB/c mice were immunized with LJM11 or BSA. Two weeks after the last immunization mice were challenged with 10^5 *L. braziliensis* promastigotes in the presence of *Lu. longipalpis* (Lb + Lulo) or *Lu. intermedia* (Lb + Linter) SGS. Production of IFN-γ (A) and IL-10 (B) by draining lymph node cells (n = 5 per group) was determined by ELISA 48 h after infection. Dot plots of one representative experiment showing frequency of CD4$^+$IFN-γ$^+$ T cells in the ear of mice (n = 5 per group) two weeks after infection (C). Experiments were repeated three times. ND, not detected.

supplemented with 10% heat inactivated fetal bovine serum (Gibco), 2 mM of L-glutamin, 100 U/mL of penicillin and 100 U/mL of streptomycin (Gibco). Homogenized samples were serially diluted into 96-wells plates containing biphasic blood agar medium (Novy-Nicolle-McNeal) and incubated for one week at 23 °C. Wells with positive parasite growth and specific dilutions were used to calculate parasite burdens (ELIDA software, 1986).

2.10. Flow cytometry

Ear cells (n = 5 per group) were obtained 48 h after the last immunization and 48 h after challenge. Ear tissues were incubated with 50 μg/ml of Liberase TL (Sigma) diluted in RPMI for one hour at 37 °C. After incubation, ear cells were macerated in complete RPMI supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) (Gibco). Pooled ear cells were placed in 96-well plates at a concentration of 5×10^6 cells/ml and unstimulated or stimulated with anti-CD3 and anti-CD28 (Ebioscience) during the first 12 h. During the last 4 h of culture, brefeldin A (BD Biosciences) was added to the cultures incubated at 37 °C, 5% CO_2. Cells were initially blocked (Fc Block CD16/CD32, 2.4G2, BD) for 30 min at 4 °C fixed and permeabilized using cytofix/cytoperm solution (BD Biosciences) and stained for 30 min at 4 °C using PerCP-Cy5.5-labeled anti-CD4 (RM 4–5) and FITC-labeled anti-TCR-b (H57-597). Intracellular staining of IFN-γ was performed with PE-labeled anti-IFN-γ (XMG 1.2) for 30 min. A minimum of 100,000 cells was acquired using a FACSAria flow cytometer (BD Biosciences). Data were analyzed using the Flow Jo software.

2.11. ELISA for cytokine detection

Lymph node cells (n = 5 per group) were obtained 48 h after challenge, macerated and plated in 96-well plates (Corning Inc. Life Sciences) at a concentration of 1×10^6 cells/ml in RPMI supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) (Gibco) unstimulated and stimulated with *L. braziliensis* promastigotes (ratio cell: parasites 1:5) for 72 h. Cytokines (IFN-γ, IL-10) detection in the supernatants was determined by ELISA according to manufactures instructions (Ebioscience).

2.12. Statistical analysis

The results were analyzed using the GraphPad Prism program-5.0 (GraphPad Software). Differences observed when comparing multiple experimental groups were analyzed by Kruskal-Wallis test (one-way ANOVA), followed by Dunns post-test. The course of the disease in the experimental and control mice were individually evaluated and the area under the curve of each of the resulting curve was calculated. P values < 0.05 were considered significant.

3. Results

3.1. Immunization of BALB/c mice with LJM11 protein produces a cellular and humoral immune response

Initially, we investigated if immunization with LJM11 was capable of inducing a specific immune response in BALB/c mice. We observed an increased production of anti-LJM11 total IgG, IgG1 and IgG2a compared to BSA controls fifteen days after the last immunization (Fig. 1A).

We also detected a LJM11-specific cellular immune response characterized by increased induration at the site of immunization (Fig. 1B). An intense inflammatory infiltrate constituted by mononuclear cells and a higher frequency of IFN-γ$^+$CD4$^+$ T cells was also detected in LJM11 immunized mice compared to controls, characterizing the

development of a Th1 environment in the skin (Fig. 1C and D).

3.2. Immunity generated by LJM11 immunization protects mice against L.braziliensis infection in the presence of Lu. longipalpis but not of Lu. intermedia SGS

To evaluate if immunity generated following LJM11 immunization resulted in protection against *L. braziliensis* infection, mice were challenged in the presence of *Lu. longipalpis* (Lbraz + Lulo) or *Lu.intermedia* (Lbraz + Linter) SGS. We observed a significant reduction in ear thickness and parasite load in the ear and draining lymph node 2, 5 and 10 weeks after challenge in the LJM11/Lbraz + Lulo group (Fig. 2A, C, D and E). Although the LJM11/Lbraz + Linter group was able to delay lesion development up to the fifth week post-challenge, parasite load in the ear and lymph node was not controlled. Mice immunized with BSA (BSA/Lbraz + Lulo and BSA/Lbraz + Linter) groups did not control parasite load in the ear and lymph node (Fig. 2C–E). However, we observed that the BSA/Lbraz + Lulo group was also able to control lesion development and disease burden similarly to the LJM11/Lbraz + Lulo group (Fig. 2B).

3.3. Immunization with LJM11 protein generates an initial predominant IFN-γ production after infection with L. braziliensis

To evaluate the immune response after *L. braziliensis* infection of mice immunized with LJM11 or BSA we investigated production of cytokines 48 h after challenge. A higher production of IFN-γ by lymph node cells from LJM11 immunized mice was detected in response to stimulation with *L. braziliensis* compared with BSA immunized groups. However, there was no significant difference between LJM11/Lbraz + Linter or LJM11/Lbraz + Lulo groups (Fig. 3A). In contrast, production of IL-10 after stimulation with *L. braziliensis* was observed only in the BSA but not in the LJM11 immunized groups (Fig. 3B).

Two weeks after infection we also detected a greater expression of CD4$^+$ IFN-γ$^+$ T cells in the BSA/Lbraz + Lulo and LJM11/Lbraz + Lulo groups (3.3% and 2.7%, respectively) when compared to BSA/Lbraz + Linter and LJM11/Lbraz + Linter groups (1.7% and 0.75%, respectively) (Fig. 3C).

4. Discussion

This study demonstrates that immunization of BALB/c mice with LJM11 salivary protein results in protection against *L. braziliensis* infection in the presence of *Lu. longipalpis* SGS where LJM11 is naturally present. However, LJM11-immunized mice were not able to control infection with *L. braziliensis* plus *Lu. intermedia* SGS.

The mechanism of protection conferred by sand fly salivary molecules correlates with the development of a DTH reaction at the site of challenge with the presence of a cellular infiltrate composed by IFN-γ$^+$CD4$^+$ T lymphocytes (Gomes et al., 2012; Teixeira et al., 2014; Oliveira et al., 2015). Here, we initially confirmed that LJM11 protein immunogenicity also extends to BALB/c mice with development of specific cellular and humoral immune responses. Immunized mice presented significant levels of anti-LJM11 total IgG, IgG1 and IgG2a compared to BSA immunized mice. In contrast, increased production of anti-LJM11 total IgG with a high IgG2a:IgG1ratio was detected in C57BL/6 immunized mice (Gomes et al., 2012). In the murine model, production of specific IgG2a has been related to a Th1 immune response and production of IFN-γ while production of IgG1 is associated to a Th2 immune response (Oliveira et al., 2006; Agallou et al., 2011). This contrasting result is probably related to the use of different mouse strains. Here, we used BALB/c mice known for a characteristic dominance of IgG1 production (Ebrahimpoor et al., 2013; Lestinova et al., 2015).

Immunization of C57BL/6 mice with LJM11 protein resulted in an increased frequency of IFN-γ$^+$CD4$^+$ T lymphocytes and protection

against *L.major* infection (Gomes et al., 2012). We show similar results, demonstrating that immunization of BALB/c mice with LJM11 protein resulted in an intense inflammatory response at the site of challenge. The predominance of mononuclear cells, accompanied by IFN-γ$^+$CD4$^+$ T lymphocytes present in the skin 48 h after the last immunization, suggests that immunization with LJM11 also generates a Th1 environment in BALB/c mice. We did not detect any IL-4 production by lymph node cells after challenge in any of the groups (data not shown). Although we cannot eliminate the possibility of other cytokines also taking part of the LJM11-induced immunity, IFN-γ and IL-10 are decisive to the control or development of infection caused by different species of *Leishmania* (Scharton-Kersten and Scott, 1995; Nylén and Sacks, 2007; Maspi et al., 2016).

Following infection with *L. braziliensis*, we observed that the LJM11/Lbraz + Lulo group was able to control lesion development and induce a significant parasite load reduction in the ear and draining lymph node. Surprisingly, we did not observe a protective response in the LJM11/Lbraz + Linter group that only presented a delay in lesion development but were not able to control parasite load. The lack of protection detected could be due to differences in the amino acid sequence between a similar yellow salivary protein present in *Lu. intermedia* saliva and *Lu. longipalpis* LJM11 salivary protein (De Moura et al., 2013). On the other hand, while we did not observe protection in the group challenged with BSA/Lbraz + Linter, a significant reduction in lesion size and initial parasite load control was detected in the BSA/Lbraz + Lulo group. This finding contrasts with previous work showing that *Lu. longipalpis* SGS exacerbate infection caused by *L. major, L. amazonensis* and *L. braziliesnis* (Theodos et al., 1991; Samuelson et al., 1991; Donnelly et al., 1998; Norsworthy et al., 2004). Importantly, they have performed infection using a large number of parasites inoculated subcutaneously, a model far from the natural transmission of *Leishmania* by the sand fly that is initiated in the skin with inoculation of a small number of metacyclic parasites and saliva (Belkaid et al., 1998; Belkaid et al., 2000; De Moura et al., 2005).

However, no difference was observed in lesion size or parasite load in control hamsters infected with *L. braziliensis* in the presence of *Lu. intermedia* or *Lu. longipalpis* SGS (Tavares et al., 2011). In a different manner, infection of hamsters with *L. infantum* plus *Lu. longipalpis* SGS did not result in increased disease burden (Gomes et al., 2008). As far as we know, the mechanism underlying the effect of *Lu. longipalpis* SGS on the course of *L. braziliensis* infection has not been described.

Here, we used laboratory-colonized *Lu. longipalpis* and wild caught *Lu. intermedia*. There is evidence showing that the immunomodulatory and protective effect of SGS from wild caught and colonized sand flies can vary due to differences in the amount and composition of salivary proteins (Laurenti et al., 2009a, 2009b; Ahmed et al., 2010 and 2016). However, hamsters immunized with wild caught *Lu. longipalpis* saliva or LJM19, a salivary molecule from *Lu. longipalpis*, were protected against *L. braziliensis* infection in the presence of wild caught *Lu. intermedia* saliva (Tavares et al., 2011). Further studies are necessary to compare the differences of sand fly saliva from wild caught and colonized sand flies from different species of sand flies.

After infection, we observed IFN-γ production by LJM11 immunized groups independently of the type of challenge. In the BSA immunized groups, there was an increased IL-10 production. Similar results were observed two weeks after challenge, where frequency of CD4$^+$IFN-γ$^+$ T cells in the lymph nodes were higher in both groups challenged with *L. braziliensis* plus *Lu. longipalpis* SGS. One possibility is that an initial production of IFN-γ detected in mice infected with *L. braziliensis* in the presence of *Lu. longipalpis* SGS could contribute to an initial control of infection leading to a delay in lesion development.

Cross-protection between different species of sand flies has been previously demonstrated. However, there is also indication that both cellular and humoral immune responses resulting from immunization with vector saliva is species-specific (Drahota et al., 2014). Immunization of mice with *Lu. longipalpis* SGS resulted in protection

against *L. amazonensis* infection but was not able to protect against infection in the presence of *P. papatasi* SGS, a more distant species of sand fly (Thiakaki et al., 2005). Cross-protection was also demonstrated in mice pre-exposed to bites of *P. papatasi* resulting in control against *L. major* infection in the presence of *P. duboscqi* or *P. papatasi* SGS (Lestinova et al., 2015). Identification of a single salivary molecule able to induce cross-protection has also been demonstrated. Immunization of hamsters with a plasmid DNA coding for LJM19, another salivary molecule from *Lu. longipalpis*, was able to induce protection against *L. braziliensis* infection in the presence of *Lu. intermedia* or *Lu. longipalpis* SGS (Tavares et al., 2011).

These results support the importance of evaluating specificity of salivary-mediated protective responses. We did not test LJM11-immunized mice against infection in the presence of SGS form other species of sand flies. Here, our results demonstrate that LJM11-induced protection might be specific to *Lu. longipalpis* SGS highlighting the importance of identifying cross-protective vaccine candidates, especially when considering endemic areas where different species of sand fly transmit the same species of *Leishmania*.

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